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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DAMHUS, Ture [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). SCHNEIDER, Palle [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). BECH, Lisbeth [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). HEINZKILL, Marion [DE/DE]; Universität Kaiserslautern, Paul-Ehrlich-Strasse 23, D-67663 Kaiserslautern (DE).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: COPRINACEAE LACCASES

(57) Abstract

The present invention relates to a method of oxidizing a substrate in a solution with a pH at or above 7, comprising contacting the substrate with an effective amount of a laccase obtainable from *Coprinaceae*.

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# COPRINACEAE LACCASES

## FIELD OF INVENTION

The present invention relates to a method of oxidizing a substrate in an alkaline solution with a laccase. The invention also relates to a detergent additive and to a detergent composition.

## BACKGROUND OF THE INVENTION

Laccases are enzymes that catalyze the oxidation of a substrate with dioxygen, reducing dioxygen to water. Such enzymes are known from microbial, plant and animal origins.

It has earlier been found that coloured substances leached from dyed fabrics could be bleached in solution by means of peroxidases or suitable oxidases. The use of peroxidases or oxidases for inhibiting dye transfer in this way is described in WO 91/05839.

Certain oxidizable substrates, e.g., metal ions and phenolic compounds such as 7-hydroxycoumarin, vanillin, and p-hydroxybenzenesulfonate, have been described as accelerators or enhancing agents able to enhance bleaching reactions (cf. e.g. WO 92/18683, WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 **26** (7), pp. 1291-1301 (cf. Table 1 in particular)). In WO 94/12621 other types of enhancing agents are disclosed, e.g., phenothiazines and phenoxazines.

It is the object of this invention to provide a laccase system with bleaching performance at high pH.

## SUMMARY OF THE INVENTION

We have unexpectedly found that laccases obtainable from Coprinaceae show excellent performance in bleaching assays at high pH.

Accordingly, the invention provides a method of oxidizing a substrate in a solution with a pH at or above 7, comprising contacting the substrate with an effective amount of

a laccase obtainable from Coprinaceae. The invention also provides use of these laccases in bleaching compositions and detergent compositions.

**BRIEF DESCRIPTION OF DRAWINGS**

The present invention is further illustrated by reference to Fig. 1 which shows the bleaching of gradually added Acid Blue 45 in phosphate/borate buffer pH 10 at 35°C; (I): Only dye addition; (II): Dye addition in the presence of Laccase; (III): Dye addition in the presence of Laccase + 10-ethylphenothiazine-4-carboxylic acid; (IV): Dye addition in the presence of Laccase + phenoxazine-10-propionic acid; the experiment conducted as described in Example 4.

**DETAILED DISCLOSURE OF THE INVENTION**

The present invention provides a method of oxidizing a substrate in a solution with a pH at or above 7, preferably in a solution with a pH at or above 8, even more preferably in a solution with a pH at or above 9, in particular in a solution with a pH around 10, comprising contacting said substrate with an effective amount of a laccase obtainable from Coprinaceae, optionally in the presence of an enhancing agent.

Laccases (EC 1.10.3.2) are oxidoreductases that function with molecular oxygen as electron acceptor. Molecular oxygen from the atmosphere will usually be present in sufficient quantity, so normally it is not necessary to add extra oxygen to the process medium.

Coprinaceae Laccases

According to the invention useful laccases that function at a high pH are obtainable from laccase-producing strains of the family Coprinaceae.

The family Coprinaceae comprises the following genera: Coprinus, Podaxis, Montagnea, Macrometrula,

Psathyrella, Panaeolina, Panaeolus, Copelandia, Anellaria, Limnoperdon, Panaeolopsis and Polyplocium.

Preferably, the laccase employed in the method of the present invention is obtainable from Coprinus, Panaeolus or Psathyrella, in particular Coprinus cinereus, Coprinus comatus, Coprinus friesii, Coprinus plicatilis, or Psathyrella condolleana, or Panaeolus papilionaceus; most preferred strain is Coprinus cinereus (IFO30116). The most preferred strain is freely available to the public from Institute of Fermentation, Osaka (IFO) under the indicated deposit number.

A strain representative of Coprinus comatus has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1995, at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, under Accession No. CBS 631.95.

A strain representative of Coprinus friesii has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1995, at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, under Accession No. CBS 629.95.

A strain representative of Coprinus plicatilis has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1995, at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, under Accession No. CBS 627.95.

A strain representative of Psathyrella condolleana has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1995, at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, under Accession No. CBS 628.95.

A strain representative of Panaeolus papilionaceus has been deposited according to the Budapest Treaty on the

International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1995, at Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, under Accession No. CBS 630.95.

The laccase according to the invention may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the laccase, in a culture medium under conditions permitting the expression of the laccase enzyme, and recovering the laccase from the culture.

Determination of Laccase Activity (LACU)

Laccase activity is determined by oxidizing syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 µM syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min reaction time.

1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 µmole syringaldazin per minute under these conditions.

Immunochemical Properties

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to I. M. Roitt; Immunology, Gower Medical Publishing (1985) and N. H. Axelsen; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), Chapters 5 and 14.

According to the invention laccases displaying immunochemical cross-reactivity with an antibody raised against

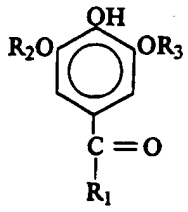
a laccase obtainable from Coprinus cinereus IFO30116 are also included.

Enhancing Agents

In oxidizing a substrate at high pH the laccases of the invention show excellent results. If additionally an enhancing agent is added, the result may be improved.

According to the invention an enhancing agent is any compound that enhances the oxidation. The enhancing agent will typically be an oxidizable compound, e.g., a metal ion or a phenolic compound such as 7-hydroxycoumarin, vanillin, or p-hydroxybenzenesulfonate, (for reference see WO 92/18683, WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 26 (7), pp. 1291-1301 (cf. Table 1 in particular)).

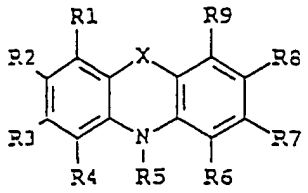
Particularly useful enhancing agents may be described by the following formula:




where $R_1$ is H, OH, $C_nH_{2n+1}$ or $OC_nH_{2n+1}$, in which n is an integer of from 1 to 10; and $R_2$ and $R_3$ are the same or different and selected from $C_mH_{2m+1}$, in which m is an integer of from 1 to 10. $R_1$, $R_2$ and $R_3$ may also contain double bonds or cyclic groups.

In particular embodiments, the enhancing agent is acetosyringone, syringaldehyde, methylsyringate or syringic acid.

Other particularly useful enhancing agents may be described by the following formula:




in which formula X represents (-O-) or (-S-), and the substituent groups $R^1$-$R^9$, which may be identical or different, independently represent any of the following radicals: hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, $C_1$-$C_{14}$-alkyl, $C_1$-$C_5$-alkoxy, carbonyl-$C_1$-$C_5$-alkyl, aryl-$C_1$-$C_5$-alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group $R^{10}$; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups $R^{10}$; and which $C_1$-$C_{14}$-alkyl, $C_1$-$C_5$-alkoxy, carbonyl-$C_1$-$C_5$-alkyl, and aryl-$C_1$-$C_5$-alkyl groups may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups $R^{10}$;

which substituent group $R^{10}$ represents any of the following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidin-1-yl, $C_1$-$C_5$-alkyl, $C_1$-$C_5$-alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy, $C_1$-$C_5$-alkyl, $C_1$-$C_5$-alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which $C_1$-$C_5$-alkyl, and $C_1$-$C_5$-alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl,

carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl;

or in which general formula two of the substituent groups $R^1$-$R^9$ may together form a group -B-, in which B represents any of the following groups: $(-CHR^{10}-N=N-)$, $(-CH=CH-)_n$, $(-CH=N-)_n$ or $(-N=CR^{10}-NR^{11}-)$, in which groups n represents an integer of from 1 to 3, $R^{10}$ is a substituent group as defined above and $R^{11}$ is defined in the same way as $R^{10}$. (It is to be understood that if the above mentioned formula comprises two or more $R^{10}$-substituent groups, these $R^{10}$-substituent groups may be the same or different).

In particular embodiments, the enhancing agent is 10-methylphenothiazine, phenothiazine-10-propionic acid, N-hydroxysuccinimide phenothiazine-10-propionate, 10-ethylphenothiazine-4-carboxylic acid, 10-ethylphenothiazine, 10-propylphenothiazine, 10-isopropylphenothiazine, methyl phenothiazine-10-propionate, 10-phenylphenothiazine, 10-allylphenothiazine, 10-(3-(4-methylpiperazin-1-yl)propyl)phenothiazine, 10-(2-pyrrolidin-1-yl-ethyl)phenothiazine, 2-methoxy-10-methylphenothiazine, 1-methoxy-10-methylphenothiazine, 3-methoxy-10-methylphenothiazine, 3,10-dimethylphenothiazine, 3,7,10-trimethylphenothiazine, 10-(2-hydroxyethyl)phenothiazine, 10-(3-hydroxypropyl)phenothiazine, 3-(2-hydroxyethyl)-10-methylphenothiazine, 3-hydroxymethyl-10-methylphenothiazine, 3,7-dibromophenothiazine-10-propionic acid, phenothiazine-10-propionamide, chlorpromazine, 2-chloro-10-methylphenothiazine, 2-acetyl-10-methylphenothiazine, 10-methylphenoxazine, 10-ethylphenoxazine, phenoxazine-10-propionic acid, 10-(2-hydroxyethyl)phenoxazine or 4-carboxyphenoxazine-10-propionic acid.

The enhancing agents may be obtained from Sigma-Aldrich, Janssen Chimica, Kodak, Tokyo Kasai Organic Chemicals, Daiichi Pure Chemicals Co. or Boehringer Mannheim; N-methylated derivatives of phenothiazine and phenoxazine may be prepared by methylation with methyliodide as described by Cornel Bodea and Ioan Silberg in "Recent Advances in the Chemistry of Phenothiazines" (Advances in heterocyclic chemistry, 1968, Vol. 9, pp. 321-460); B. Cardillo & G. Casnati in Tetrahedron, 1967,

Vol. 23, p. 3771. Phenothiazine and phenoxazine propionic acids may be prepared as described in J. Org. Chem. 15, 1950, pp. 1125-1130. Hydroxyethyl and hydroxypropyl derivatives of phenothiazine and phenoxazine may be prepared as described by G. Cauquil in Bulletin de la Society Chemique de France, 1960, p.1049.

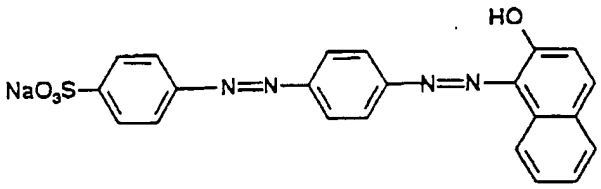
The enhancing agent may be present in concentrations of from 0.01 to 500 μM, preferably in concentrations of from 0.1 to 250 μM.

Industrial Applications

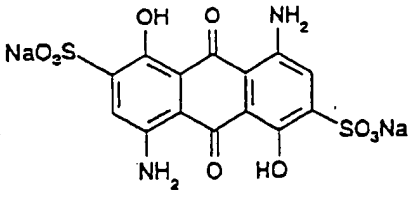
In a preferred embodiment, the method of the invention finds application for bleaching of a dye or dyes in solutions.

The dye may be a synthetic dye such as an azo or an anthraquinone dye, or a natural or nature-identical dye. Examples of dyes are Acid Red 151, Acid blue 45, Direct Blue 1, and indigo carmine, where Acid Red 151, Acid blue 45 and Direct Blue 1 have the following formulas:

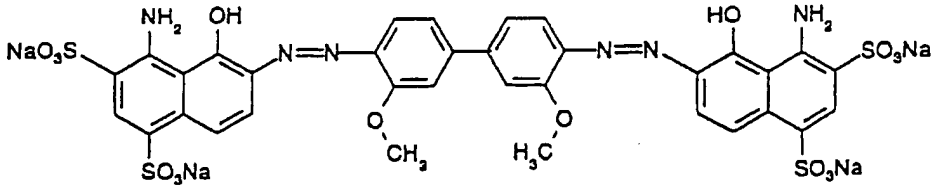
Acid red 151:



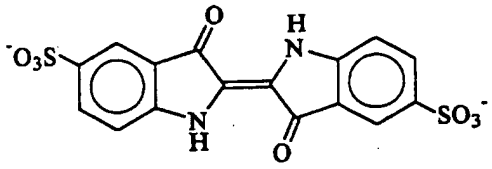

Acid blue 45:



Direct blue 1:



and indigo carmine has the following formula:



In another embodiment, the method of the invention finds application for dye transfer inhibition, e.g., during processing of dyed textiles (cf. e.g. WO 92/18687) or during laundering (cf. e.g. WO 91/05839).

Accordingly, in a specific embodiment, the invention provides a method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are rinsed together or washed together in a wash liquor, the method comprising treatment of the wash liquor with a laccase obtainable from Coprinaceae in the presence or absence of an enhancing agent.

In another embodiment, the method of the invention finds application in treatment of waste water, e.g., waste water from the chemical or pharmaceutical industry, from dye manufacturing, from dye-works or from the textile industry.

According to the invention the laccase may be present in concentrations of from 0.001-500 LACU/liter, preferably in concentrations of from 0.5-100 LACU/liter.

Detergent Compositions

According to the invention, a laccase of the invention may be added as a component of a detergent composition. As such, it may be included in the detergent composition in the form of a detergent additive. The detergent composition as well as the detergent additive may additionally comprise one or more other enzymes, such as proteases, lipases, amylases, cutinases, cellulases and other oxidoreductases than laccases, e.g., peroxidases and hydrogen peroxide generating oxidases.

In a specific aspect, the invention provides a detergent additive. The enzymes may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separated additive or a combined additive, can be formulated e.g. as granulates, liquids, slurries, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, slurries, or protected enzymes.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as amylases, lipases, cutinases, proteases, cellulases, and other oxidoreductases than laccases, e.g., peroxidases and hydrogen peroxide generating oxidases.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a $H_2O_2$ source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 7 - 12% |
| Alcohol ethoxysulfate (e.g. $C_{12-18}$ alcohol, 1-2 EO) or alkyl sulfate (e.g. $C_{16-18}$) | 1 - 4% |
| Alcohol ethoxylate (e.g. $C_{14-15}$ alcohol, 7 EO) | 5 - 9% |
| Sodium carbonate (as $Na_2CO_3$) | 14 - 20% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 2 - 6% |
| Zeolite (as $NaAlSiO_4$) | 15 - 22% |

| | |
|---|---|
| Sodium sulfate (as $Na_2SO_4$) | 0 - 6% |
| Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$) | 0 - 15% |
| Sodium perborate (as $NaBO_3.H_2O$) | 11 - 18% |
| TAED | 2 - 6% |
| Carboxymethylcellulose | 0 - 2% |
| Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) | 0 - 3% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach) | 0 - 5% |

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 6 - 11% |
| Alcohol ethoxysulfate (e.g. $C_{12-18}$ alcohol, 1-2 EO or alkyl sulfate (e.g. $C_{16-18}$) | 1 - 3% |
| Alcohol ethoxylate (e.g. $C_{14-15}$ alcohol, 7 EO) | 5 - 9% |
| Sodium carbonate (as $Na_2CO_3$) | 15 - 21% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 1 - 4% |
| Zeolite (as $NaAlSiO_4$) | 24 - 34% |
| Sodium sulfate (as $Na_2SO_4$) | 4 - 10% |
| Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$) | 0 - 15% |
| Carboxymethylcellulose | 0 - 2% |
| Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) | 1 - 6% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. suds suppressors, perfume) | 0 - 5% |

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 5 - 9% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO) | 7 - 14% |
| Soap as fatty acid (e.g. $C_{16-22}$ fatty acid) | 1 - 3% |
| Sodium carbonate (as $Na_2CO_3$) | 10 - 17% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 3 - 9% |
| Zeolite (as $NaAlSiO_4$) | 23 - 33% |
| Sodium sulfate (as $Na_2SO4$) | 0 - 4% |
| Sodium perborate (as $NaBO_3.H_2O$) | 8 - 16% |
| TAED | 2 - 8% |
| Phosphonate (e.g. EDTMPA) | 0 - 1% |
| Carboxymethylcellulose | 0 - 2% |
| Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) | 0 - 3% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. suds suppressors, perfume, optical brightener) | 0 - 5% |

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 8 - 12% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO) | 10 - 25% |
| Sodium carbonate (as $Na_2CO_3$) | 14 - 22% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 1 - 5% |
| Zeolite (as $NaAlSiO_4$) | 25 - 35% |
| Sodium sulfate (as $Na_2SO_4$) | 0 - 10% |
| Carboxymethylcellulose | 0 - 2% |

| | |
|---|---|
| Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) | 1 - 3% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. suds suppressors, perfume) | 0 - 5% |

5) An aqueous liquid detergent composition comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 15 - 21% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO or $C_{12-15}$ alcohol, 5 EO) | 12 - 18% |
| Soap as fatty acid (e.g. oleic acid) | 3 - 13% |
| Alkenylsuccinic acid ($C_{12-14}$) | 0 - 13% |
| Aminoethanol | 8 - 18% |
| Citric acid | 2 - 8% |
| Phosphonate | 0 - 3% |
| Polymers (e.g. PVP, PEG) | 0 - 3% |
| Borate (as $B_4O_7$) | 0 - 2% |
| Ethanol | 0 - 3% |
| Propylene glycol | 8 - 14% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener) | 0 - 5% |

6) An aqueous structured liquid detergent composition comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 15 - 21% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) | 3 - 9% |
| Soap as fatty acid (e.g. oleic acid) | 3 - 10% |
| Zeolite (as $NaAlSiO_4$) | 14 - 22% |
| Potassium citrate | 9 - 18% |
| Borate (as $B_4O_7$) | 0 - 2% |
| Carboxymethylcellulose | 0 - 2% |
| Polymers (e.g. PEG, PVP) | 0 - 3% |
| Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800 | 0 - 3% |
| Glycerol | 0 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners) | 0 - 5% |

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Fatty alcohol sulfate | 5 - 10% |
| Ethoxylated fatty acid monoethanolamide | 3 - 9% |
| Soap as fatty acid | 0 - 3% |
| Sodium carbonate (as $Na_2CO_3$) | 5 - 10% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 1 - 4% |
| Zeolite (as $NaAlSiO_4$) | 20 - 40% |
| Sodium sulfate (as $Na_2SO_4$) | 2 - 8% |
| Sodium perborate (as $NaBO_3.H_2O$) | 12 - 18% |
| TAED | 2 - 7% |

| | |
|---|---|
| Polymers (e.g. maleic/acrylic acid copolymer, PEG) | 1 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. optical brightener, suds suppressors, perfume) | 0 - 5% |

8) A detergent composition formulated as a granulate comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 8 - 14% |
| Ethoxylated fatty acid monoethanolamide | 5 - 11% |
| Soap as fatty acid | 0 - 3% |
| Sodium carbonate (as $Na_2CO_3$) | 4 - 10% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 1 - 4% |
| Zeolite (as $NaAlSiO_4$) | 30 - 50% |
| Sodium sulfate (as $Na_2SO_4$) | 3 - 11% |
| Sodium citrate (as $C_6H_5Na_3O_7$) | 5 - 12% |
| Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG) | 1 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. suds suppressors, perfume) | 0 - 5% |

9) A detergent composition formulated as a granulate comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 6 - 12% |
| Nonionic surfactant | 1 - 4% |
| Soap as fatty acid | 2 - 6% |
| Sodium carbonate (as $Na_2CO_3$) | 14 - 22% |
| Zeolite (as $NaAlSiO_4$) | 18 - 32% |
| Sodium sulfate (as $Na_2SO_4$) | 5 - 20% |
| Sodium citrate (as $C_6H_5Na_3O_7$) | 3 - 8% |
| Sodium perborate (as $NaBO_3.H_2O$) | 4 - 9% |
| Bleach activator (e.g. NOBS or TAED) | 1 - 5% |

| | |
|---|---|
| Carboxymethylcellulose | 0 - 2% |
| Polymers (e.g. polycarboxylate or PEG) | 1 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. optical brightener, perfume) | 0 - 5% |

10) An aqueous liquid detergent composition comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 15 - 23% |
| Alcohol ethoxysulfate (e.g. $C_{12-15}$ alcohol, 2-3 EO) | 8 - 15% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) | 3 - 9% |
| Soap as fatty acid (e.g. lauric acid) | 0 - 3% |
| Aminoethanol | 1 - 5% |
| Sodium citrate | 5 - 10% |
| Hydrotrope (e.g. sodium toluensulfonate) | 2 - 6% |
| Borate (as $B_4O_7$) | 0 - 2% |
| Carboxymethylcellulose | 0 - 1% |
| Ethanol | 1 - 3% |
| Propylene glycol | 2 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners) | 0 - 5% |

11) An aqueous liquid detergent composition comprising

| | |
|---|---|
| Linear alkylbenzenesulfonate (calculated as acid) | 20 - 32% |
| Alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) | 6 - 12% |
| Aminoethanol | 2 - 6% |

| | |
|---|---|
| Citric acid | 8 - 14% |
| Borate (as $B_4O_7$) | 1 - 3% |
| Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer) | 0 - 3% |
| Glycerol | 3 - 8% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners) | 0 - 5% |

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap) | 25 - 40% |
| Nonionic surfactant (e.g. alcohol ethoxylate) | 1 - 10% |
| Sodium carbonate (as $Na_2CO_3$) | 8 - 25% |
| Soluble silicates (as $Na_2O$, $2SiO_2$) | 5 - 15% |
| Sodium sulfate (as $Na_2SO_4$) | 0 - 5% |
| Zeolite (as $NaAlSiO_4$) | 15 - 28% |
| Sodium perborate (as $NaBO_3.4H_2O$) | 0 - 20% |
| Bleach activator (TAED or NOBS) | 0 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. perfume, optical brighteners) | 0 - 3% |

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by ($C_{12}$-$C_{18}$) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| ($C_{12}$-$C_{18}$) alkyl sulfate | 9 - 15% |
| Alcohol ethoxylate | 3 - 6% |
| Polyhydroxy alkyl fatty acid amide | 1 - 5% |
| Zeolite (as $NaAlSiO_4$) | 10 - 20% |
| Layered disilicate (e.g. SK56 from Hoechst) | 10 - 20% |
| Sodium carbonate (as $Na_2CO_3$) | 3 - 12% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 0 - 6% |
| Sodium citrate | 4 - 8% |
| Sodium percarbonate | 13 - 22% |
| TAED | 3 - 8% |
| Polymers (e.g. polycarboxylates and PVP) | 0 - 5% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors) | 0 - 5% |

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| ($C_{12}$-$C_{18}$) alkyl sulfate | 4 - 8% |
| Alcohol ethoxylate | 11 - 15% |
| Soap | 1 - 4% |
| Zeolite MAP or zeolite A | 35 - 45% |
| Sodium carbonate (as $Na_2CO_3$) | 2 - 8% |
| Soluble silicate (as $Na_2O,2SiO_2$) | 0 - 4% |
| Sodium percarbonate | 13 - 22% |
| TAED | 1 - 8% |
| Carboxymethyl cellulose | 0 - 3% |
| Polymers (e.g. polycarboxylates and PVP) | 0 - 3% |
| Enzymes (calculated as pure enzyme protein) | 0.0001 - 0.1% |
| Minor ingredients (e.g. optical brightener, phosphonate, perfume) | 0 - 3% |

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The laccases of the invention may be incorporated at an enzyme protein level conventionally employed for other enzymes in detergents. It is at present contemplated that, in detergent compositions of the invention, the laccases may be added at a level corresponding to 0.00001-5 mg, preferably at a level corresponding to 0.0001-1 mg (calculated as pure enzyme protein) per liter of wash liquor.

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

**EXAMPLE 1**

Bleaching of Direct Blue 1 (DB1) using Coprinaceae laccases with various enhancing agents at pH 5.5-8.5.

Bleaching of the dye Direct Blue 1 at various pH values was conducted using a laccase obtained from Coprinus comatus, Coprinus friesii, Coprinus plicatilis, Panaeolus papilionaceus or Psathyrella condolleana and one of the following enhancing agents:

PPT (phenothiazine-10-propionic acid)

PPO (phenoxazine-10-propionic acid)

methylsyringate.

The above mentioned strains were fermented in the following way:

The strains were inoculated on PDA agar plates (PDA: 39 g/l potato dextrose agar) and grown at 26°C for 3 days. Shake flasks were then inoculated with 6-8 small squares (~0.5 cm x 0.5 cm) of agar containing mycelium and fermented for 3-10 days at 26°C and 200 rpm using the following medium:

| | Deposit no. | Medium | Growth |
|---|---|---|---|
| Coprinus comatus | CBS 631.95 | A | 10 days |
| Coprinus friesii | CBS 629.95 | A | 3 days |
| Panaeolus papilionaceus | CBS 630.95 | A | 10 days |
| Psathyrella condolleana | CBS 628.95 | B | 7 days |
| Coprinus plicatilis | CBS 627.95 | A | 8 days |

Media:

| | | |
|---|---|---|
| A: | soja meal | 30 g/l |
| | maltodextrin | 15 g/l |
| | bacto peptone | 5 g/l |
| | pluronic | 0.2 g/l |

| | | |
|---|---|---|
| B: | potato meal | 50 g/l |
| | barley meal | 25 g/l |
| | BAN 800MG* | 0.025 g/l |
| | Na-caseinate | 5 g/l |
| | crushed soja | 10 g/l |
| | Na2HPO4, 12 H2O | 4.5 g/l |
| | Pluronic | 0.05 ml/l |

* BAN 800MG obtainable from Novo Nordisk A/S.

After fermentation the culture broths were centrifugated and the supernatants were used in the tests described below.

The bleaching rate of DB1 was determined using the following conditions:

| | Final concentration |
|---|---|
| 400 µl 50 mM Britton-Robinson buffer*, (pH 5.5, 7.0, and 8.5 respectively), | 20 mM |
| 200 µl DB1 ~ 3.0 Abs. Units (610 nm) | 0.6 ($A_{610nm}$) |
| 200 µl 50 µM enhancing agent | 10 µM |
| 200 µl laccase | 4 LACU/l at pH 5.5 and at pH 7.0; 20 LACU/l at pH 8.5. |

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 ml thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was followed spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1, with readings every 5 sec. for a period of 5 minutes. The initial bleaching rate was determined from the first linear part of the absorbance curve.

The following results were obtained with PPT:

-ΔmAbs/minute

| Laccase: pH: | 5.5 | 7.0 | 8.5 |
|---|---|---|---|
| C. comatus | 68 | 17 | 3 |
| C. friesii | 221 | 44 | 36 |
| Pan. papilionaceus | 91 | 25 | 9 |
| Ps. condolleana | 88 | 36 | 25 |
| C. plicalitis | 75 | 33 | 0 |

The following results were obtained with PPO:

-ΔmAbs/minute

| Laccase: pH: | 5.5 | 7.0 | 8.5 |
|---|---|---|---|
| C. comatus | 102 | 44 | 4 |
| C. friesii | 122 | 80 | 116 |
| Pan. papilionaceus | 120 | 67 | 47 |
| Ps. condolleana | 114 | 114 | 72 |
| C. plicalitis | 106 | 83 | 23 |

The following results were obtained with methyl-syringate:

-ΔmAbs/minute

| Laccase: pH: | 5.5 | 7.0 | 8.5 |
|---|---|---|---|
| C. comatus | 33 | 23 | 2 |
| C. friesii | 40 | 55 | 61 |
| Pan. papilionaceus | 16 | 19 | 18 |
| Ps. condolleana | 45 | 54 | 43 |
| C. plicalitis | 42 | 39 | 14 |

**EXAMPLE 2**

Bleaching of Direct Blue 1 (DB1) using Coprinus cinereus laccase with/without enhancing agents at pH 5.5-8.5.

Bleaching of the dye Direct Blue 1 at various pH values was conducted using Coprinus cinereus laccase and one of the following enhancing agents:
None
PPT (phenothiazine-10-propionic acid)
MPT (10-methylphenothiazine)
PPO (phenoxazine-10-propionic acid)
MPO (10-methylphenoxazine)
EPC (10-ethylphenothiazine-4-carboxylic acid)
acetosyringone
syringaldehyde
methylsyringate
ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)).

The laccase was obtained in the following way:
Coprinus cinereus (IFO 30116) was inoculated from a PDA agar slant (PDA: 39 g/l potato dextrose agar) into a 100 ml shake flask containing medium A (Medium A is described in Example 1). The culture was cultivated for 6 days at 26°C and 100 rpm. A 10-liter fermentor containing medium A was inoculated with the 100 ml culture broth. The fermentation ran for 6 days at 26°C and 100 rpm. The culture broth was filtrated and concentrated by ultrafiltration. Further purification was carried out using hydrophobic interaction chromatography followed by anionic exchange chromatography. This process resultated in a preparation with a laccase activity of 3.6 LACU/ml. The estimated purity was >80% on a protein basis.

The bleaching rate of DB1 was determined using the following conditions:

| | Final concentration |
|---|---|
| 400 µl 50 mM Britton-Robinson buffer*, (pH 5.5, 7.0 and 8.5 respectively), | 20 mM |
| 200 µl DB1 ~ 3.0 Abs. Units (610 nm) | 0.6 ($A_{610nm}$) |
| 200 µl 50 µM enhancing agent | 10 µM |
| 200 µl C. cinereus laccase | 4 LACU/l |

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 ml thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was followed spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1, with readings every 5 sec. for a period of 5 minutes. The initial bleaching rate was determined from the first linear part of the absorbance curve.

The following results were obtained:

| Enhancing agent | -ΔmAbs/minute | | |
|---|---|---|---|
| pH: | 5.5 | 7.0 | 8.5 |
| none | 13 | 5 | 3 |
| PPT | 74 | 74 | 24 |
| MPT | 43 | 30 | 1 |
| PPO | 121 | 104 | 48 |
| MPO | 83 | 74 | 30 |
| EPC | 184 | 122 | 57 |
| aceto-syringone | 28 | 94 | 50 |
| syring-aldehyde | 29 | 79 | 28 |
| methyl-syringate | 20 | 94 | 57 |
| ABTS | 84 | 76 | 24 |

**EXAMPLE 3**

Bleaching of Direct Blue 1 (DB1) using Coprinus cinereus laccase and acetosyringone

Bleaching of the dye Direct Blue 1 at various pH values was conducted using Coprinus cinereus laccase and the enhancing agent acetosyringone.

The laccase was obtained as described in Example 2. Acetosyringone was obtained from Aldrich.

The bleaching rate of DB1 was determined using the following conditions:

| | Final concentration |
|---|---|
| 400 µl 50 mM Britton-Robinson buffer*, (pH 4, 5, 6, 7, and 8 respectively), | 20 mM |
| 200 µl DB1 ~ 3.0 Abs. Units (610 nm) | 0.6 ($A_{610nm}$) |
| 200 µl 50 µM acetosyringone | 10 µM |
| 200 µl C. cinereus laccase | 13 LACU/l |

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 cm thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was detected spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1. After 5 sec. bleaching was followed for 4 minutes.

The following results were obtained:

| pH | Initial DB1 bleaching (-ΔmAbs/ min) (% of pH 7-value) |
|---|---|
| 4 | 18 % |
| 5 | 13 % |
| 6 | 35 % |
| 7 | 100 % |
| 8 | 69 % |

It can be seen from the results given above that the optimum bleaching is achieved at pH around 7, but the system also shows a significant bleaching at pH 8.

**EXAMPLE 4**

Bleaching of gradually added Acid Blue 45 with Coprinus cinereus Laccase with and without enhancing agents

Ideally, dye transfer inhibition systems for laundry applications should be tested in a real wash where dyed fabrics give off dyes to the wash solution as a result of the combined action of the detergent, temperature and mechanical agitation taking place.

To simulate such a process, however, a magnetically stirred beaker was used as the reaction vessel and dye was added gradually from a stock solution (using a Metrohm 725 dosimat). The solution was monitored spectrophotometrically using a a Zeiss multichannel spectrometer (MCS) equipped with a fibre-optics immersion probe.

Stock solutions of the enhancing agents were prepared either in a suitable water/ethanol mixture or by first dissolving the enhancing agent in a small amount of dimethylformamide and then diluting with a pH 10.0 phosphate/borate buffer. Stock solutions of the dye Acid Blue 45 were made with water.

The laccase was obtained as described in Example 2.

The following conditions were used in the experiment:

Temperature: 35°C
Medium and pH: 50 mM/50 mM phosphate/borate buffer at pH 10
Enhancing agent (when applicable): 10 µM
Laccase: 0.04 LACU/ml
Dye addition program: linear addition at rates of ca 0.34 abs/40 min, referring to the absorbance of the dye at its maximum absorbance wavelength (590 nm for Acid Blue 45).

The following enhancing agents were tested: phenoxazine-10-propionic acid and 10-ethylphenothiazine-4-carboxylic acid.

Fig. 1 shows the results of the bleaching tests. The following symbols are used: (I): Only dye addition; (II): Dye addition in the presence of Laccase; (III): Dye addition in the presence of Laccase + 10-ethylphenothiazine-4-carboxylic acid; (IV): Dye addition in the presence of Laccase + phenoxazine-10-propionic acid.

It can be seen from Fig. 1 that the tested laccase performs well even without enhancing agents at pH 10; but the bleaching effect is enhanced by phenoxazine-10-propionic acid and 10-ethylphenothiazine-4-carboxylic acid.

**EXAMPLE 5**

Dye Transfer Inhibition Using Coprinus cinereus Laccase

A small-scale experiment was carried out in which clean cotton test pieces were washed together with dyed fabrics bleeding dye into the wash solution, the experiment conducted in the absence and in the presence of laccase and enhancing agent.

After wash, the Hunter colour difference between the above mentioned cotton pieces and clean cotton pieces (washed in the absence of bleeding fabrics) was measured and taken as a measure of the degree of dye transfer resulting from the wash.

Materials used:

Bleeding fabrics dyed with Acid Red 151 (AR 151) or Direct Blue 1 (DB1).

Clean white cotton (bleached, no optical brightener added).

Liquid detergent and powder detergent as typically met in the North American market place; both detergents contained no bleaching system.

Coprinus cinereus laccase, obtained as described in Example 2.

Washing procedure:

The washing processes were carried out in beakers with magnetical stirring at 35°C for 15 min., after which the test fabrics were rinsed thoroughly in tap water and air-dried overnight in the dark before the Hunter readings were taken by using a Datacolor Elrephometer 2000 reflectance spectrometer.

Treatment 1 was in each case a wash with laccase at a level of 40 LACU/l with the enhancing agent 10-ethylphenothiazine-4-carboxylic acid at a level of 10 µM.

Treatment 2 was in each case a wash with laccase at a level of 40 LACU/l with the enhancing agent acetosyringone at a level of 10 µM.

The following results were obtained:

Wash in liquid detergent solution (2 g/l, water hardness 6°dH) at pH 8.5:

| | Hunter colour difference (delta E) with respect to white, washed cotton | |
|---|---|---|
| | Cotton washed with AR 151 | Cotton washed with DB 1 |
| Wash with no laccase system | 12 | 26 |
| Treatment 1 | 9 | 3 |
| Treatment 2 | 1 | 7 |

Wash in powder detergent solution (1 g/l, water hardness 6°dH) at pH 10.0:

| | Hunter colour difference (delta E) with respect to white, washed cotton | |
|---|---|---|
| | Cotton washed with AR 151 | Cotton washed with DB 1 |

| | | |
|---|---|---|
| Wash with no laccase system | 21 | 29 |
| Treatment 1 | 17 | 13 |
| Treatment 2 | 4 | 8 |

Typical significant differences in the delta E readings are 2-3 units, so the data reflect significant reduction of dye transfer with the laccase treatments relative to the treatment with no laccase system.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, lines 11-16.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

| Date of deposit | Accession Number |
|---|---|
| 16 August 1995 | CBS 631.95 |

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

| For receiving Office use only | For International Bureau use only |
|---|---|
| [X] This sheet was received with the international application | [ ] This sheet was received by the International Bureau on: |
| Authorized officer | Authorized officer |

Form PCT/RO/134 (July 1992)

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line s 17-22.

**B. IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

| Date of deposit | Accession Number |
|---|---|
| 16 August 1995 | CBS 629.95 |

**C. ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

**D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

**E. SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, lines 23-29.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

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| 16 August 1995 | CBS 627.95 |

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 30-36.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution
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Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

| Date of deposit | Accession Number |
|---|---|
| 16 August 1995 | CBS 628.95 |

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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A. The indications made below relate to the microorganism referred to in the description on page 3 and 4, line 37-38 (page 3) and. 1-5 (page 4)

**B. IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☐

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Netherland

| Date of deposit | Accession Number |
|---|---|
| 16 August 1995 | CBS 630.95 |

**C. ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

**D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

**E. SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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**CLAIMS**

1. A method of oxidizing a substrate in a solution with a pH at or above 7, comprising contacting the substrate with an effective amount of a laccase obtainable from Coprinaceae.

2. A method according to claim 1, wherein the laccase is obtainable from Coprinus, Podaxis, Montagnea, Macrometrula, Psathyrella, Panaeolina, Panaeolus, Copelandia, Anellaria, Limnoperdon, Panaeolopsis or Polyplocium.

3. A method according to claim 2, wherein the laccase is obtainable from Coprinus cinereus.

4. A method according to claim 3, wherein the laccase is obtainable from Coprinus cinereus IFO30116, or is a laccase displaying immunochemical cross-reactivity with an antibody raised against a laccase obtainable from Coprinus cinereus IFO30116.

5. A method according to claim 1, wherein an effective amount of laccase is in the range of from 0.001 LACU/liter to 500 LACU/liter.

6. A method according to claims 1-5, additionally comprising contacting the substrate with an effective amount of an enhancing agent.

7. A method according to claim 6, wherein the enhancing agent is phenothiazine or phenoxazine or a derivative one of these.

8. A method according to claim 7, wherein the enhancing agent is selected from the group consisting of phenothiazine-10-propionic acid, 10-methylphenothiazine,

phenoxazine-10-propionic acid, 10-methylphenoxazine and 10-ethylphenothiazine-4-carboxylic acid.

9. A method according to claim 6, wherein the enhancing agent is acetosyringone, syringaldehyde or methyl-syringate.

10. A method according to any of claims 1-9, in which said method is a method for bleaching of a dye or dyes in solution.

11. A method according to any of claims 1-10, in which said method is a method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor.

12. A method according to any of claims 6-9, in which the enhancing agent is added at the beginning of or during the process.

13. A method according to any preceding claim, in which the level of enhancing agent added is in the range of from 0.01-500 μM, preferably in the range of from 0.1-250 μM.

14. A detergent additive for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed and/or rinsed together in a wash liquor characterized by comprising

a) a laccase obtainable from Coprinaceae

b) optionally an enhancing agent.

15. A detergent additive according to claim 14, wherein the laccase is obtainable from Coprinus, in particular from Coprinus cinereus.

16. A detergent additive according to claims 14-15, wherein the laccase is obtainable from Coprinus cinereus IFO30116, or is a laccase displaying immunochemical cross-

reactivity with an antibody raised against a laccase obtainable from Coprinus cinereus IFO30116.

17. A detergent additive according to claims 14-16, wherein the enhancing agent is phenothiazine or phenoxazine or a derivative one of these.

18. A detergent additive according to claim 17, wherein the enhancing agent is selected from the group consisting of phenothiazine-10-propionic acid, 10-methylphenothiazine, phenoxazine-10-propionic acid, 10-methylphenoxazine and 10-ethylphenothiazine-4-carboxylic acid.

19. A detergent additive according to claims 14-16, wherein the enhancing agent is acetosyringone, syringaldehyde or methylsyringate.

20. A detergent additive according to any of claims 14-19, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

21. A detergent composition comprising a laccase obtainable from Coprinaceae, a surfactant and optionally an enhancing agent.

22. A detergent composition according to claim 21, wherein the laccase is obtainable from Coprinus, in particular from Coprinus cinereus.

23. A detergent composition according to claims 21-22, wherein the laccase is obtainable from Coprinus cinereus IFO30116 or is a laccase displaying immunochemical cross-reactivity with an antibody raised against a laccase derived from Coprinus cinereus IFO30116.

24. A detergent composition according to claims 21-23, wherein the enhancing agent is phenothiazine or phenoxazine or a derivative one of these.

25. A detergent composition according to claim 24, wherein the enhancing agent is selected from the group consisting of phenothiazine-10-propionic acid, 10-methylphenothiazine, phenoxazine-10-propionic acid, 10-methylphenoxazine and 10-ethylphenothiazine-4-carboxylic acid.

26. A detergent composition according to claim 23, wherein the enhancing agent is acetosyringone, syringaldehyde or methylsyringate.

27. A detergent composition according to any of claims 21-26, which further comprises one or more other enzymes, in particular a protease, a lipase, an amylase, a cutinase, a cellulase and/or an another oxidoreductase than laccase, e.g., a peroxidase and/or a hydrogen peroxide generating oxidase.

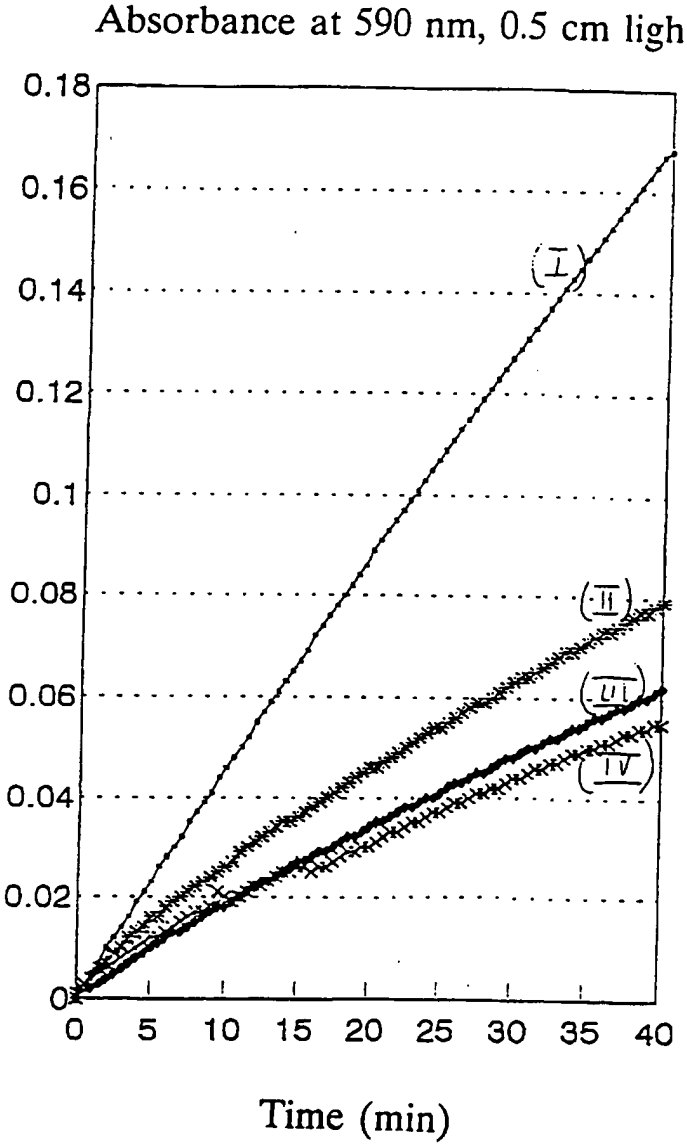
Absorbance at 590 nm, 0.5 cm light path
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 5 10 15 20 25 30 35 40
Time (min)
(I)
(II)
(III)
(IV)

FIG. 1

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00344

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/02, C11D 3/386 // C12N 9/02, C12R 1:645
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C11D, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9313193 A1 (NOVO NORDISK A/S), 8 July 1993 (08.07.93), page 6, line 10 - page 8, line 14, claims 1,9,22 -- | 1-27 |
| X | WO 9105839 A1 (NOVO NORDISK A/S), 2 May 1991 (02.05.91), page 6, line 6 - line 24; page 7, line 16 - line 21, claims 5,22 -- | 1-27 |
| A | Patent Abstracts of Japan, Vol 9,No 324, C-320, abstract of JP, A, 60-156385 (Kyowa Hakko Kogyo K.K.), 16 August 1985 (16.08.85) -- | 1-23 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 28 November 1995 | 30.11.95 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br><br>Yvonne Siösteen<br>Telephone No. +46 8 782 25 00 |

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**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/DK 95/00344

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 9218683 A1 (NOVO NORDISK A/S), 29 October 1992 (29.10.92), page 3, line 7 - line 20; page 5, line 23 - line 26, and the claims<br>-- | 1-23 |
| A | WO 9218687 A1 (NOVO NORDISK A/S), 29 October 1992 (29.10.92), page 5, line 8; page 5, line 20 - line 21; page 7, line 6 - line 8<br>--<br>-------- | 1-23 |

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## INTERNATIONAL SEARCH REPORT

Information on patent family members

30/10/95

International application No.
PCT/DK 95/00344

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A1- 9313193 | 08/07/93 | EP-A- 0617734 | 05/10/94 |
| | | JP-T- 7504694 | 25/05/95 |
| WO-A1- 9105839 | 02/05/91 | AT-T- 108484 | 15/07/94 |
| | | AU-B- 646645 | 03/03/94 |
| | | AU-A- 6515790 | 16/05/91 |
| | | AU-A- 6516090 | 16/05/91 |
| | | CA-A- 2067748 | 14/04/91 |
| | | CN-A- 1051600 | 22/05/91 |
| | | DE-D,T- 69010691 | 16/03/95 |
| | | EP-A,B- 0495836 | 29/07/92 |
| | | SE-T3- 0495836 | |
| | | EP-A,B- 0497794 | 12/08/92 |
| | | SE-T3- 0497794 | |
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| | | JP-T- 5500899 | 25/02/93 |
| | | JP-T- 5503542 | 10/06/93 |
| | | TR-A- 26687 | 15/05/95 |
| | | US-A- 5273896 | 28/12/93 |
| | | WO-A- 9105858 | 02/05/91 |
| WO-A1- 9218683 | 29/10/92 | NONE | |
| WO-A1- 9218687 | 29/10/92 | EP-A- 0580707 | 02/02/94 |
| | | JP-T- 6506731 | 28/07/94 |
| | | US-A- 5356437 | 18/10/94 |

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